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Expression Profile of microRNA-150 in Human Papillomavirus-Induced Lesions of K14-HPV16 Transgenic Mice

Dissertação de Candidatura ao Grau de Mestre em Oncologia – Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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Informação Técnica

TÍTULO:
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Abbreviations

A

AGO – Argonaute

Akt – Protein kinase B

C

cDNA – Complementary deoxyribonucleic acid

CIN – Cervical intraepithelial neoplasia

CIS – Carcinoma *in situ*

CKIs – Cyclin-dependent kinase inhibitors

COX – Cyclooxygenase

CSF-1R – Colony-stimulating factor-1 receptor

CSS – Cervical squamous cell carcinoma

Ct – Threshold cycle

D

DGCR8 – DiGeorge syndrome critical region gene 8

DNA – Deoxyribonucleic acid

dsDNA – Double-stranded deoxyribonucleic acid

E

E6-AP – E6-associated protein

EBV – Epstein-Barr Virus

EGFR – Epidermal growth-factor receptor

H

HBV – Hepatitis B Virus

HCV – Hepatitis C Virus

HDACs – Histone deacetylases

H&E – Haematoxylin and eosin

HHV-8 – Human Herpes Virus 8

HPV – Human Papillomavirus

hTERT – Human telomerase reverse transcriptase

HTLV-1 – Human T-cell lymphotropic virus type 1

HTMs – Histone methyltransferases

I

IARC – International Agency for Research on Cancer

K

K14 – Keratin-14

KSHV – Kaposi's Sarcoma-Associated Herpesvirus

L

LCR – Long control region

M

MHC – Major histocompatibility complex

mRNA – Messenger ribonucleic acid

miR – microRNA

miRISC – microRNA-induced silencing complex

miRNAs – microRNAs

N

ncRNAs – Non-coding ribonucleic acids

NF-kB – Nuclear factor-kappa B

O

OncomiRs – Oncogenic microRNAs

ORF – Open reading frame

P

PDGFβR – Platelet-derived growth factor-β receptor

PDK-1 – Kinase 3-phosphoinositide-dependent kinase-1

pRB – Retinoblastoma protein

Pre-miRNAs – Precursor-miRNAs

Pri-miRNAs – Primary-miRNAs

PVs – Papillomaviruses

Q

qPCR – Quantitative real-time polymerase chain reaction

R

RNA – Ribonucleic acid

RSV – Rous sarcoma virus

S

SCCHN – Squamous cell carcinoma of the head and neck

snoRNA – Small nucleolar ribonucleic acid

SNPs – Single nucleotide polymorphisms

SRCIN1 – SRC Kinase Signaling Inhibitor 1

T

TNFα – Tumor necrosis factor alpha

TRBP – Trans-activation response RNA-binding protein

U

UTR – Untranslated region

V

VEGF – Vascular Endothelial Growth Factor

W

WHO – World Health Organization

X

XPO5 – Exportin-5

Table of Contents

Figures Index

Tables Index

Abstract

Abstract

Introduction: High-risk human papillomavirus (HPV) infection is one of the major causes of infection-related cancers worldwide. MicroRNAs (miRNAs) are a family of non-coding RNAs (ncRNAs), whose function is to modulate the expression of genes involved in essential cellular processes, and which are dysregulated in many cancers. MiRNA-150 expression profile in HPV-induced lesions remains elusive. K14-HPV16 transgenic mice express the early genes of HPV16 in basal keratinocytes, leading to the development of hyperplastic and dysplastic skin lesions and squamous cell carcinomas, thus becoming a representative model of HPV-induced cancers. Celecoxib is a cyclooxygenase-2 (COX-2) inhibitor and several studies have been confirming that this pharmacological agent is able to suppress tumor growth.

Material and Methods: In order to evaluate the expression of miR-150 in HPV-induced lesions, we performed qPCR to measure the miR-150 levels in wild-type mice ($HPV^{-/-}$), in skin lesions of K14-HPV16 transgenic mice ($HPV^{+/-}$) with no treatment and in skin lesions of $HPV^{+/-}$ mice treated with two doses of celecoxib. To assess the type of lesion present in each mouse, histological evaluation was also performed.

Results: 24-26 weeks old $HPV^{+/-}$ mice showed epidermal hyperplasia and dysplasia, but HPV+/- mice at 28-30 weeks old presented higher incidence of dysplasia. MiR-150 was upregulated in lesions of $HPV^{+/-}$ mice when compared with normal skin from $HPV^{-/-}$ mice. MiR-150 was also overexpressed in lesions of $HPV^{+/-}$ mice at 28-30 weeks of age when compared with lesions of $HPV^{+/-}$ mice at 24-26 weeks of age. In lesions of $HPV^{+/-}$ mice treated with 124 mg/kg/day of celecoxib, miR-150 expression decreased significantly when compared with lesions of $HPV^{+/-}$ mice without treatment and the incidence of epidermal dysplasia was not observed. In $HPV^{+/-}$ mice treated with 75 mg/kg/day of celecoxib, statistical significance was not observed.

Conclusions: The results obtained suggest that miR-150 is overexpressed in HPV-induced lesions in this model and its expression seems to increase with lesions progression. Celecoxib may modulate the expression of miR-150 in HPV-induced lesions in a seemingly dose-dependent manner. Given the influence of miR-150 in the progression of HPV-induced lesions, further studies are needed.

Resumo

Resumo

Introdução: A infeção por papilomavírus humano (HPV) de alto risco é uma das principais causas de cancro relacionado com infeções em todo o mundo. Os microRNAs (miRNAs) são uma família de RNAs não codificantes (ncRNAs), cuja função é modular a expressão de genes envolvidos em processos celulares essenciais, e cuja expressão se pode encontrar desregulada em vários cancros. O perfil de expressão do miRNA-150 em lesões induzidas por HPV ainda não é bem conhecido. Os murganhos transgénicos K14-HPV16 expressam os genes da região *early* do HPV16 nos queratinócitos basais, o que leva ao desenvolvimento de lesões hiperplásicas e displásicas na pele e a carcinomas de células escamosas, tornando-se assim um modelo representativo de neoplasias associadas ao HPV. O celecoxib é um inibidor da ciclocoxigenase-2 (COX-2) e vários estudos têm confirmado que este fármaco é capaz de impedir o crescimento tumoral.

Material e Métodos: Para realizar este estudo, foi usado qPCR para avaliar os níveis de expressão do miR-150 em murganhos do tipo selvagem ($HPV^{-/-}$), em lesões de murganhos transgénicos K14-HPV16 ($HPV^{+/-}$) sem tratamento e em lesões de murganhos $HPV^{+/-}$ tratados com baixa e alta dose de celecoxib. Para determinar o tipo de lesão presente em cada murganho foi realizada a análise histológica de cada amostra.

Resultados: Em murganhos $HPV^{+/-}$ com 24-26 semanas de idade foram observadas lesões hiperplásicas e displásicas, mas os murganhos $HPV^{+/-}$ com 28-30 semanas apresentam maior incidência de displasia. Em lesões de murganhos $HPV^{+/-}$ verificou-se um aumento da expressão do miR-150 em comparação com murganhos $HPV^{-/-}$. O MiR-150 também se encontra sobreexpresso em lesões de murganhos $HPV^{+/-}$ com 28-30 semanas de idade quando comparado com lesões de murganhos $HPV^{+/-}$ com 24-26 semanas de idade. Em lesões de murganhos $HPV^{+/-}$ tratados com 124 mg/kg/dia de celecoxib, a expressão do miR-150 diminuiu significativamente quando comparado com lesões de murganhos $HPV^{+/-}$ sem tratamento e a incidência de displasia não foi observada. Em murganhos $HPV^{+/-}$ tratados com 75 mg/kg/day de celecoxib não foram encontradas diferenças estatisticamente significativas.

Conclusão: Os resultados obtidos sugerem que neste modelo o miR-150 é sobreexpresso em lesões induzidas pelo HPV e que a sua expressão parece aumentar com a progressão das lesões. O celecoxib parece modular a expressão do miR-150 em lesões provocadas pelo HPV de um modo dependente da dose. Dada a influência do miR-150 na progressão das lesões induzidas pelo HPV, estudos futuros são necessários.

Introduction

Introduction

1. Cancer

1.1. Definition and Epidemiology

Cancer is a group of diseases characterized by genetic and/or epigenetic changes that alter the expression or function of key genes responsible for regulating fundamental cellular processes [1,2]. The dysregulation of the different cellular signaling pathways results in the formation of abnormal cells with an uncontrolled growth and capable of invading tissues and metastasize to distant sites [1,2].

Cancer has revealed itself as a growing public health problem worldwide. The incidence of cancer is increasing and it is the leading cause of death worldwide due to population growth and ageing as well as increasing prevalence of risk factors such as smoking, obesity, physical inactivity and changing of reproductive patterns [3]. According to GLOBOCAN, in 2012 an estimated 14.1 million new cases of cancer and 8.2 million deaths occurred worldwide [3]. In Portugal, over 24 000 deaths due to oncologic diseases were observed in 2012 [4]. For the next decades, an increase of 38% is expected in the overall number of deaths in Portuguese population, with a higher increase in men than in women [4].

1.2. Biology

The process that leads a normal tissue to transform into a neoplastic tissue is called tumorigenesis, which is a multistep process that requires the accumulation of successive alterations that confer selective advantage to the neoplastic cell (also known as hallmarks of cancer) [5,6]. These hallmarks include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [5,6]. Due to advances in cancer research, additional hallmarks have emerged such as capability to modify or reprogram cellular metabolism, capacity to evade immunological destruction in particular by T and B lymphocytes, macrophages and natural killer cells, genomic instability and tumor-promoting inflammation [6].

The majority of genetic and epigenetic changes that give rise to these hallmarks necessary for tumorigenesis are not inherited, but arise spontaneously as a consequence of DNA damage by chemical, physical or biological carcinogens [7].

Nowadays, the biological carcinogens are a well characterized group which includes infectious agents such as a few helminths (*Clonorchis sinensis*, *Opisthorchis viverrini* and *Schistosoma haematobium*), bacteria (*Helicobacter pylori*) and some viruses [8,9].

2. Tumor Viruses

The virus-tumor association was established in the middle 20th century, with the identification of some viruses as strong risk factors for specific cancers [10]. It is estimated that 15%-20% of human cancers worldwide are attributable to viral infection and the majority occurs in less developed regions [9,11–13].

The first evidence that viruses can cause cancer came in 1908, when it was shown by Vilhelm Ellermann and Olaf Bang that a cell-free filtrate of chicken leukemia cells could transmit leukemia when inoculated into healthy chickens. However, leukemia was not considered as a neoplastic disease until the 1930s, therefore the filterable agent present in the leukemia cell extracts was not recognized as a tumor virus and the importance of this discovery went largely unnoticed [14]. A few years later, in 1911, Peyton Rous showed that a cell-free filtrate from spindle cell sarcoma derived from a chicken could transmit the disease to healthy chickens, indicating that the tumor was due to a filterable agent, later known as Rous sarcoma virus (RSV) (Figure 1) [14,15]. Thereby, this discovery, awarded with the Noble Prize in Physiology or Medicine in 1966, led to the recognition of a new paradigm in cancer research: the virus-induced cancer [13,14].

Figure 1 *- Peyton Rous' protocol for inducing sarcomas in chickens. Adapted from [16].*

Viruses can contribute to carcinogenesis by direct and/or indirect mechanisms [17]. In direct carcinogenesis, the virus induces expression of oncogenes causing a tumor phenotype in the infected cell [17]. In indirect carcinogenesis, the virus is not conditioned to exist within the cell that forms the tumor and acts through chronic inflammation that persistently damage the surrounding tissues, or by inducing immunosuppression which reduces or eliminates immune surveillance [17].

Since the first evidences of virus-induced cancer, diverse molecular, epidemiologic and serologic studies have uncovered the carcinogenic potential of further viruses [11]. Up to now, viruses that are proven to be associated with human cancer are listed in Table 1.

Table 1 *- Human Tumor Viruses*

Virus	Human Cancers	Carcinogenic mechanism	References
High-risk Human Papillomavirus (HPV)	Cervical, Head and neck, Vagina, Vulva, Anus, Penis	Direct carcinogenesis	[9,13,18]
Epstein-Barr Virus (EBV)	Burkitt's lymphoma, Nasopharyngeal carcinoma, Hodgkin lymphoma, Gastric carcinoma	Direct carcinogenesis	[9,13,19]
Human T-cell lymphotropic virus type 1 (HTLV-1)	Adult T-cell leukemia	Indirect carcinogenesis via immune suppression	[9,13,20]
Kaposi's Sarcoma-Associated Herpesvirus (KSHV) / Human Herpes Virus 8 (HHV-8)	Kaposi sarcoma, Primary effusion lymphoma	Direct carcinogenesis	[9,13,21]
Hepatitis B Virus (HBV)	Hepatocellular carcinoma	Direct carcinogenesis or indirect carcinogenesis via chronic inflammation	[9,13,17]
Hepatitis C Virus (HCV)	Hepatocellular carcinoma, Non-Hodgkin lymphoma	Direct carcinogenesis or indirect carcinogenesis via chronic inflammation	[9,13,17]

Currently, one of the best established etiological agent of human cancer is the Human papillomavirus (HPV). Epidemiological studies implicated high-risk HPV infection as one of the major causes of infection-related cancers worldwide and particularly as the main cause of cervical cancer. Cervical cancer is the second leading cause of cancer-related mortality in women worldwide, with approximately 85% of these deaths occurring in developing countries (Figure 2) [22–24].

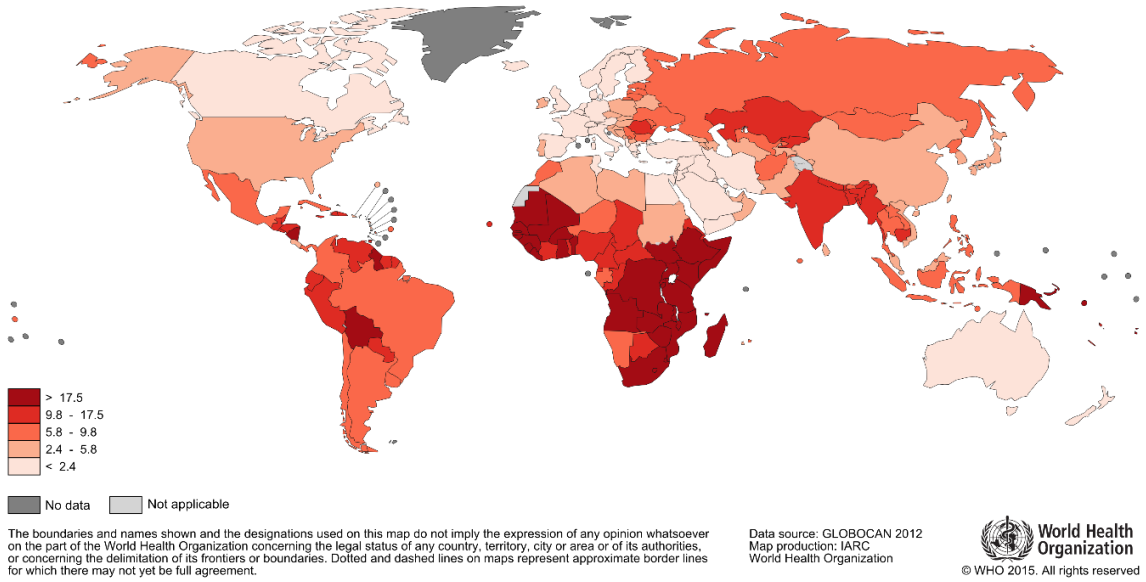

Figure 2 *- Estimated cervical cancer mortality worldwide in 2012. Estimated age-standardised rate per 100,000. Adapted from: GLOBOCAN (http://globocan.iarc.fr/).*

Finally, and most importantly, bearing in mind the impact of these agents in the development of cancer, the discovery and study of these viruses and its carcinogenic mechanisms is extremely important for prevention, prognostic and treatment of virus-induced cancers [11,13,17].

3. Human Papillomavirus

Papillomaviruses (PVs) are non-enveloped, double-stranded DNA (dsDNA) and icosahedral-shaped viruses with 50–60 nm of diameter, that belong to the family *Papillomaviridae* [25–27]. This group infects not only mammals but also birds and reptiles [28].

In humans, more than 150 HPV types have been identified, that are contained within five evolutionary groups with different epithelial tropisms and life cycle strategies: Alpha-papillomavirus, Beta-papillomavirus, Gamma-papillomavirus, Mu-papillomavirus and Nu-papillomavirus (Figure 3) [27,28]. The Beta and Gamma HPV types cause chronic unapparent infections and produce virions without causing apparent damage to the host [28]. On the other hand, the Alpha-papillomavirus includes the low-risk types associated with genital warts, and the high-risk types that are associated with the development of certain types of cancer [28].

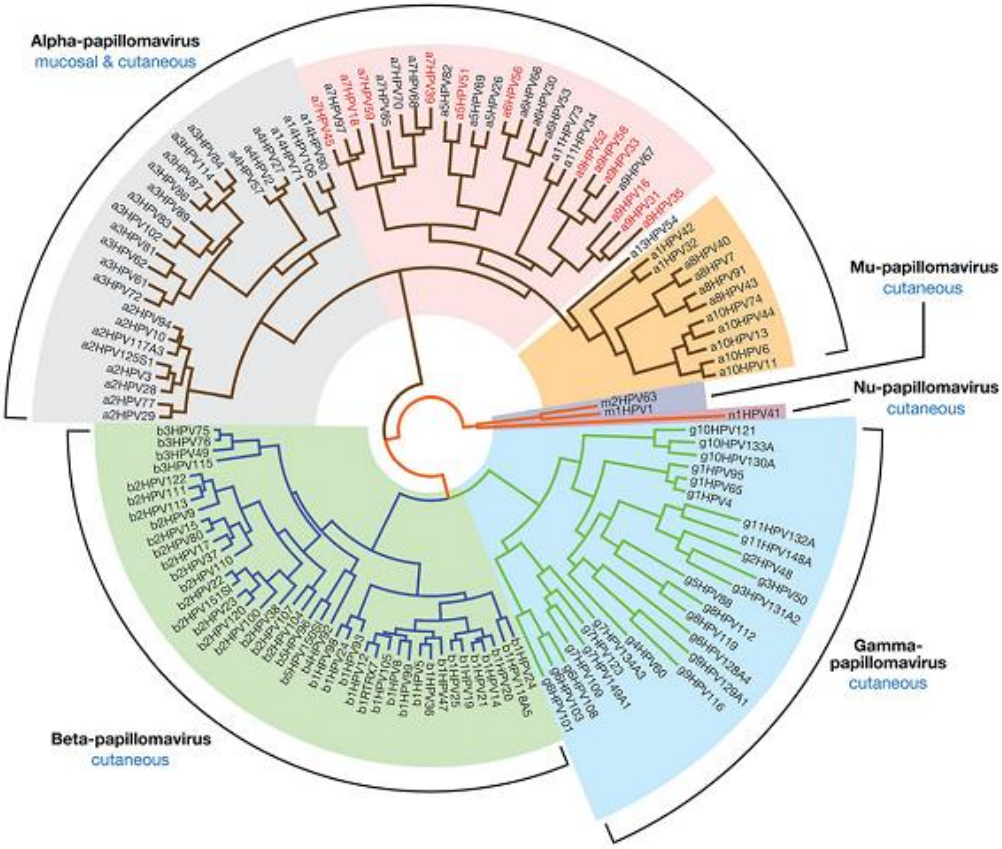

Figure 3 *- Evolutionary Relationship between the five HPV groups. Adapted from [27].*

The association between high-risk HPV and cancer was discovered by Harald zur Hausen in 1974, through the detection of HPV DNA in cervical cancer and genital wart biopsies, which gave him the Noble Prize in Physiology or Medicine in 2008 [29].

Currently, it is recognized that approximately 5% of all cancers worldwide are attributed to high-risk HPVs, according them the classification of group 1 human carcinogens by the International Agency for Research on Cancer (IARC) [18]. The infection by HPV is commonly transmitted by sexual contact and affects men and women [30,31]. The World Health Organization (WHO) defined thirteen high-risk types of HPV (16, 18, 3, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) which are considered as the causal agents for cancers of the uterine cervix, vagina, vulva, anus, penis and some head and neck cancers [18,28].

The most prevalent high-risk HPV type in the worldwide population is HPV16 followed by HPV18 [24]. Moreover, in cervical cancer HPV DNA is present at least in 99,9% of the cases, being that HPV16 is present in more than 50% and HPV18 in approximately 15% [18,25]. In head and neck squamous cell carcinomas, HPV16 is found in up to 90% of HPV-positive cases [32].

HPV16 has the typical genome organization of the high-risk Alpha-papillomaviruses (Figure 4) [28]. Its genome contains around 8000 base pairs that comprise a long control region (LCR) and eight open reading frames (ORFs) (*E6*, *E7*, *E1*, *E2*, *E4*, *E5*, *L1* and *L2*) that are necessary for the different stages of the virus life cycle [28,30,33].

Specifically, the LCR contains binding sites for cellular transcription factors as well as for E1 and E2 proteins [28]. The coding sequences have been classified as early (E) containing the early genes (*E6*, *E7*, *E1*, *E2*, *E4* and *E5*) and late (L) containing the late genes namely the *L1* and *L2* genes [28].

The early genes are responsible for the regulation of the viral replication: E1 function as the regulator of episomal viral DNA replication; E2 is a transcriptional regulator of *E6* and *E7*; E4 is involved in cytoskeletal reorganization; E5 stimulates cell growth and E6 and E7 are the transforming proteins [30,33]. Conversely, the late genes encode the viral capsid proteins for packaging newly synthesized virions [33]. Moreover, PAE indicates the position of the early polyadenylation site and PAL indicates the position of the late polyadenylation site, while PE (or p97) and PL (or p670) represent the early and late promoter, respectively (Figure 4) [28].

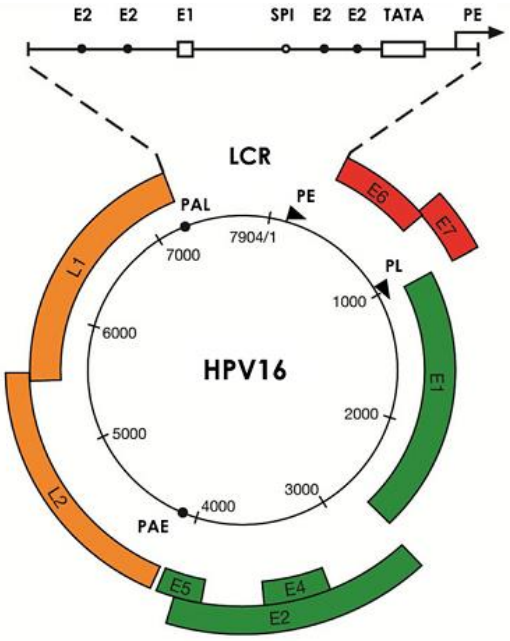

Figure 4 *- dsDNA genome organization of HPV16. Adapted from [28].*

3.1. HPV Life Cycle

HPV is a non-lytic virus that infects the stratified squamous epithelium and the completion of its life cycle depends on epithelial squamous differentiation [26,34]. In fact, the regulation of viral gene expression occurs while the infected basal cell migrates towards the epithelial surface (Figure 5) [34].

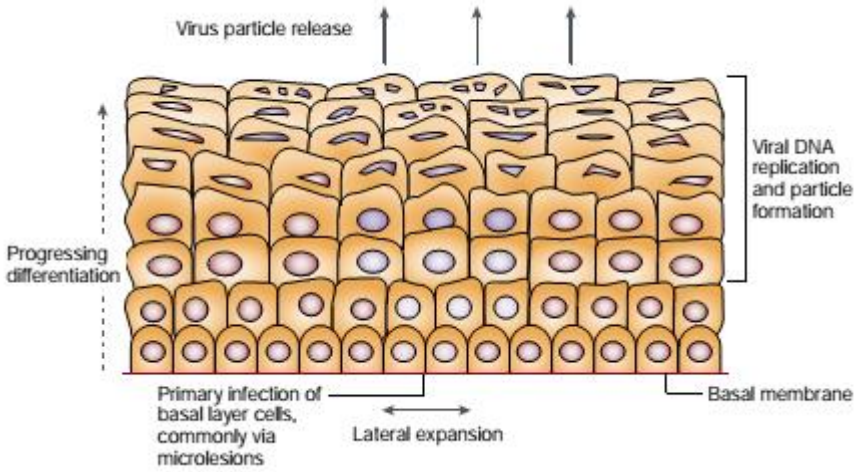

Figure 5 *- HPV life cycle. Adapted from* [30].

Initial infection requires the presence of microlesions in the epithelium that allow the infectious virions to access the epithelial basal stem cells, which are the only actively

dividing cells in the epithelial layer [27,28,32,34]. Additionally, active cell division due to wound healing is necessary for viral genome entry into the cell nucleus [27]. Once in these basal cells, the viral genome is maintained as a low copy number episome (often quoted as 200 copies per cell) [27]. However, while the infected cells divide, viral DNA is distributed between both daughter cells: one daughter cell continues to divide in the basal layer and the other migrates away from the basal layer and initiates cell differentiation. Therefore, HPV DNA replicates to a high copy number only in terminally differentiated cells near the epithelial surface [32,35].

In the mid or upper epithelial layers occurs the amplification of the viral genome and its package into infectious particles [34]. Since terminally differentiated epithelial cells are normally unable to support DNA synthesis, E6 and E7 proteins play an essential role in driving S-phase re-entry to allow viral genome amplification [27,32]. Finally, the completion of the HPV life cycle involves expression of two structural proteins: the major coat protein (L1) and minor coat protein (L2) which will allow the genome packaging [27,34].

3.2. HPV-induced Carcinogenesis

In the absence of the control of HPV infection by immunological intervention, lesions caused by high-risk HPV may persist and in some instances progress to cancer through a multistep process that develops from premalignant intraepithelial hyperplastic and dysplastic lesions to carcinoma *in situ* (CIS), and subsequently to invasive squamous cell carcinoma (Figure 6) [30,34,36].

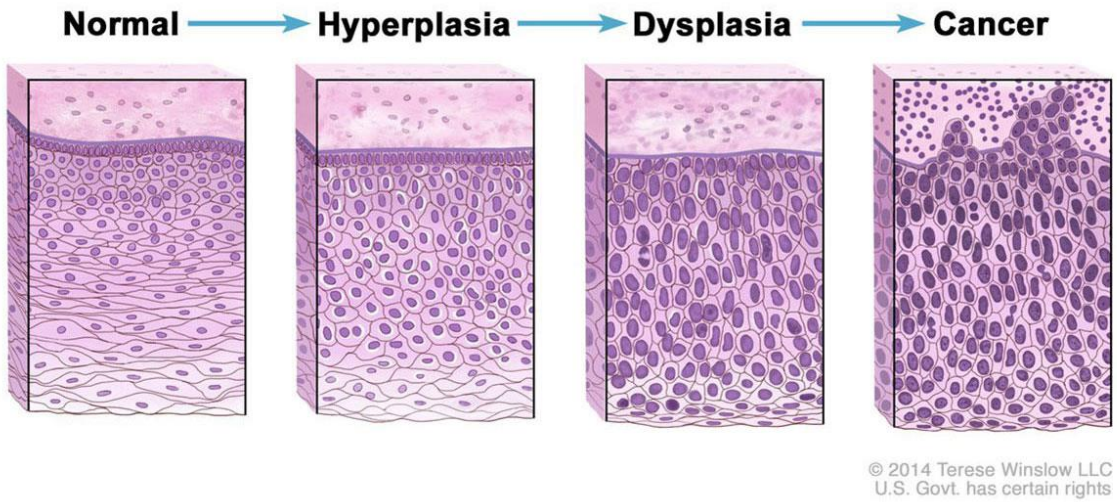

Figure 6 *– Stages of cancer development. Adapted from: National Cancer Institute (http://www.cancer.gov)*

In high-risk HPV, E6 and E7 proteins play an oncogenic role due to their capacity to form specific complexes with tumor suppressor proteins inducing proliferation, immortalization and malignant transformation in the basal and parabasal cell layers [27,37].

The tumor suppressor p53 is one of the targets of E6 oncoprotein [38]. p53 is a DNA-binding protein expressed in response to DNA damage or unscheduled induction of DNA replication, resulting in cell cycle arrest or apoptosis [37]. Particularly, E6 binds to p53 protein through a cellular ubiquitin-ligase, the E6-associated protein (E6-AP), which recruits the ubiquitin complex initiating p53 proteolysis [37]. Degradation of p53 causes chromosomal instability (with mutational consequences), inactivation of Bax (a pro-apoptotic Bcl-2 protein) and enhancement of foreign DNA integration into the host-cell genome [37,39]. Moreover, E6 can also upregulate telomerase activity by the activation of the catalytic subunit of human telomerase (hTERT) [37,38]. Telomerase is an enzyme that replicates telomeric DNA at the ends of chromosomes and whose activity is absent in normal somatic cells. Thus, when telomeres get shorter through successive cell divisions, it is initiated the natural pathway that leads to senescence and cell death [37]. However, the upregulation of telomerase activity by E6 oncoprotein allows cell immortalization and long-term tumor growth [40]. Additionally, E6 inhibits degradation of SRC-family kinases by E6-AP, stimulating mitotic activity (Figure 7) [37].

On the other hand, E7 oncoprotein is an inactivator of the retinoblastoma protein (pRB) [38]. In normal cells, the active form of pRB is hypophosphorylated and bound to E2F transcription factors [37]. This complex recruits chromatin remodeling factors (histone deacetylases (HDACs) and histone methyltransferases (HMTs)) and inhibits the transcription of genes required for the S-phase [1]. Therefore, cells remain in G1 phase of cell cycle [1]. Upon phosphorylation, pRB becomes inactive and dissociates from E2F allowing it to act as a transcriptional activator of S-phase genes leading to cell cycle progression [1,37]. When E7 oncoprotein is associated with hypophosphorylated pRB, the formation of the pRB/E2F complex is inhibited, and E2F promotes the cell cycle progression [37]. E7 also interacts with p170 and p130, two other members of the pRb family, whose function is also to downregulate E2F transcription [37]. It is also reported that E7 stimulates the S-phase genes cyclin E and cyclin A, interacts with cyclin-kinase complexes and annuls the inhibitory activities of cyclin-dependent kinase inhibitors (CKIs), such as $p21^{CIP-1/WAF-1}$ and $p27^{KIP-1}$ [37]. In addition, E7 can induce genomic instability by inducing centriole amplification (Figure 7) [37]. Furthermore, E7 reduces the total major histocompatibility complex (MHC) abundance at the cell surface, which is expected to contribute to immune escape [27].

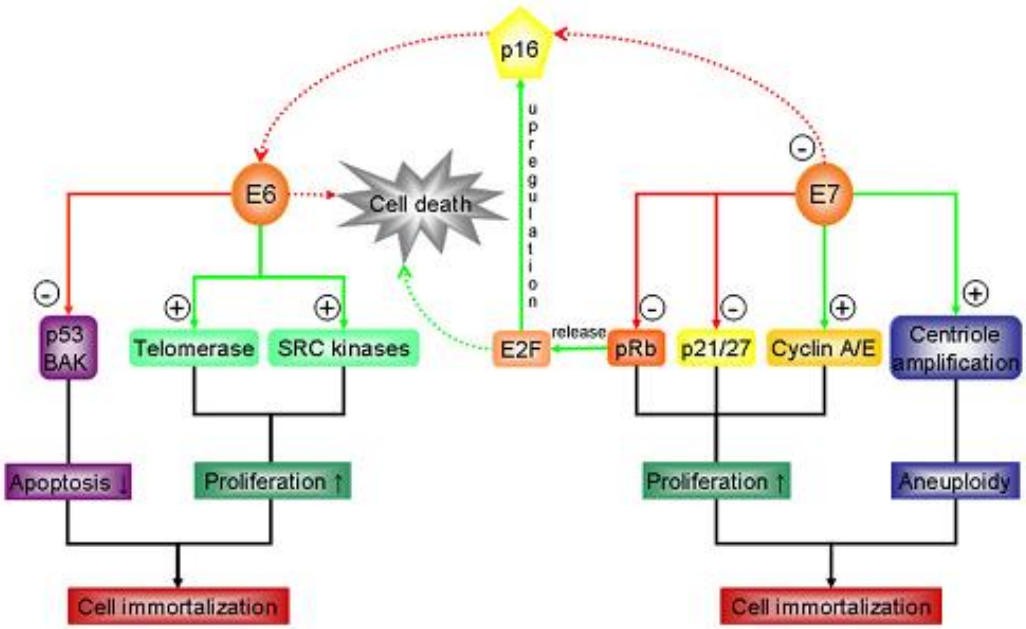

Figure 7 *- Cellular interactions of E6 and E7 oncoproteins for induction of cell immortalization. Adapted from [37].*

In addition to E6 and E7, the papillomaviral E5 proteins have been shown to contribute to tumor progression through the stimulation of cell growth by forming a complex with the epidermal growth-factor receptor (EGFR), the platelet-derived growth factor-β receptor (PDGFβR) and the colony-stimulating factor-1 receptor (CSF-1R) [30]. Likewise, it is reported that E5 prevents apoptosis following DNA damage and also contributes to immune escape by interfering with the classical MHC class I process, compromising the display of viral peptides at the surface of the infected epithelial cell [27,30].

For 40 years, diverse biological and epidemiological studies have been demonstrating the association of cervical cancer and persistent HPV infection. However, it is important to highlight that although high-risk HPV infection is necessary and the main driver of cervical carcinogenesis, it is not sufficient to complete the neoplastic transformation [18]. In fact, recent studies have significantly enhanced the complexity of HPV-induced carcinogenesis with the discovery of non-coding RNAs (ncRNAs) as important players at regulating a variety of pathways involving proliferation, differentiation and immune surveillance [41,42].

4. MicroRNAs

For normal cells, it is crucial that protein synthesis occurs in a controlled manner [43]. Therefore, the expression of a gene may be regulated at the genetic and/or epigenetic level [43]. The epigenetic regulation comprises three fields: DNA methylation, histone modifications and expression of ncRNAs [43]. NcRNAs comprehend different classes of RNAs that do not encode a protein, in which are included the microRNAs (miRNAs), a well-conserved family of approximately 22 nucleotides in length [44,45].

In 1993, the first miRNA, lin-4, was identified in the nematode *Caenorhabditis elegans* during developmental timing studies. Since then, remarkable advances in the characterization of miRNAs have demonstrated that these molecules are a prevalent class of regulatory RNAs, whose function is to modulate gene expression [45,46].

Numerous studies have indicated that miRNAs play a role in most of the fundamental biological processes such as development, proliferation, apoptosis, hematopoietic lineage differentiation and tumorigenesis [45,47–49]. Moreover, it is estimated that miRNAs control 30%-90% of the human and mouse transcriptome [50]. Actually, miRNA expression and regulation are dynamic processes, and it is postulated that each miRNA regulates approximately 100 different messenger RNAs (mRNAs) and that more than 10.000 mRNAs appear to be directly regulated by miRNAs [51].

MiRNAs biogenesis initiates in the nucleus, passes through many post-transcriptional modifications and ends in the cytoplasm (Figure 8) [45]. MiRNAs genes are mostly transcribed by RNA polymerase II, generating primary-miRNAs (pri-miRNAs) with a hairpin RNA structure [45]. These pri-miRNAs are processed by the microprocessor which is composed by enzymes Drosha (a RNase III endonuclease) and DiGeorge syndrome critical region gene 8 (DGCR8, a double-stranded RNA-binding protein also known as Pasha), into approximately 70-nucleotide precursor-miRNAs (pre-miRNAs) [45,52]. Specifically, the RNase domains of Drosha cleave the 5’ and 3’ arms of the pri-miRNAs and DGCR8 directly and stably interacts with the pri-miRNAs and determines the precise cleavage site [53].

After nuclear processing, pre-miRNAs are exported to the cytoplasm via Exportin-5 (XPO5) in complex with Ran-GTP [53]. In the cytoplasm, the RNase III Dicer in association with the double-stranded RNA-binding protein (TRBP) processes pre-miRNAs into their mature form by excising a double-stranded RNA of approximately 22 nucleotides in length (miRNA:miRNA* duplex) from the pre-miRNA hairpin [52,53]. Only the strand with a relatively lower stability of base-pairing at the 5’-end of the miRNA:miRNA* duplex is loaded together with Argonaute (AGO) proteins into the miRNA-induced silencing complex (miRISC), that guides the single-stranded miRNAs to their target mRNAs [53,54].

The function of miRNAs is to negatively regulate gene expression by post-transcriptional mechanisms [52]. The 5'-end region (also known as 'seed site') of the miRNA is important for the stability and correct loading of the miRNA into the miRISC complex and for binding to the target mRNA [52,55]. MiRNAs mainly recognize the 3'-untranslated region (UTR) in their target mRNAs, but recent studies have reported that miRNAs can also bind to the ORF or the 5'-UTR [55]. When a miRNA binds to its target mRNA with imperfect complementarity translational repression is induced [52]. Conversely, miRNAs that bind to their target mRNA with perfect or nearly perfect complementarity induce target mRNA cleavage (Figure 8) [52].

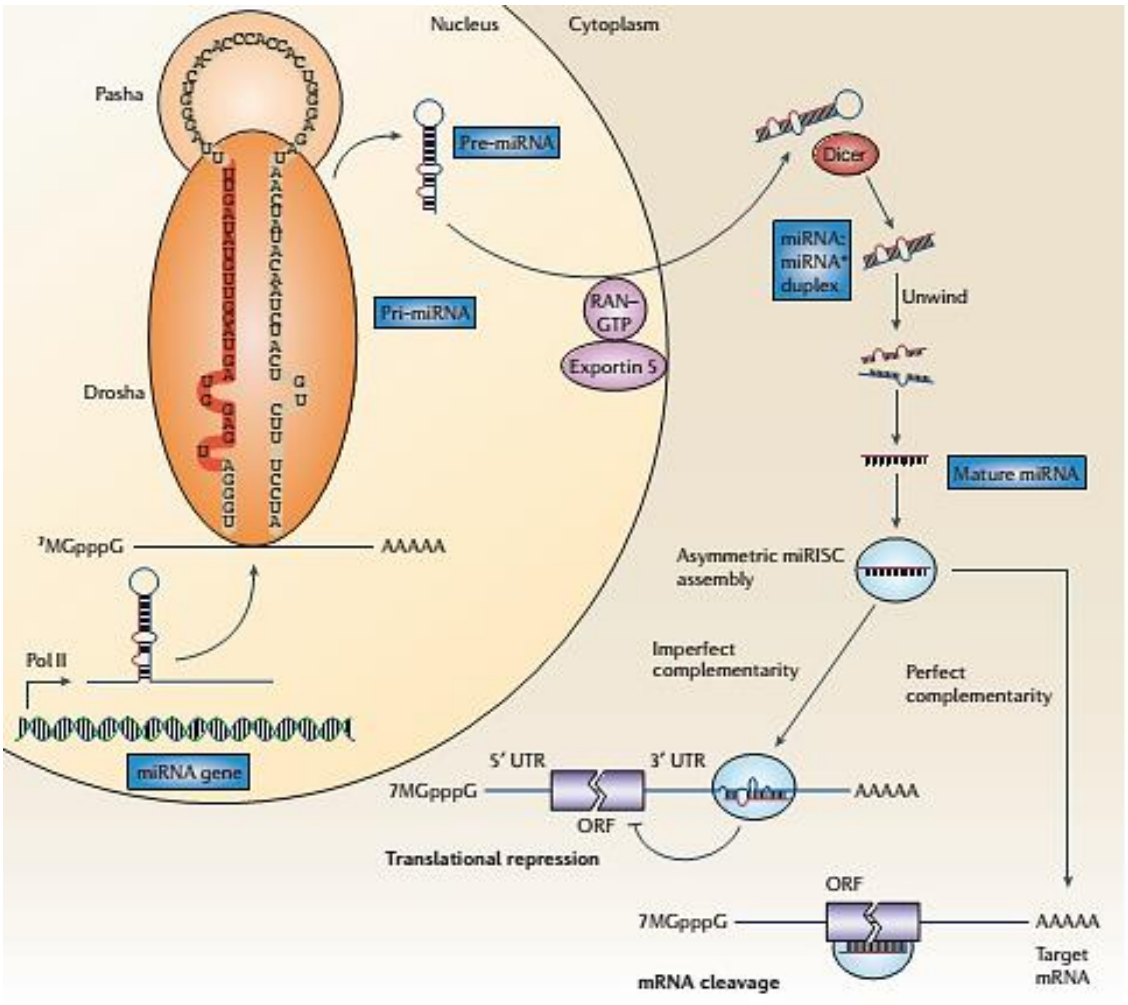

Figure 8 *- MiRNAs biogenesis and function. Adapted from* [52].

4.1. MicroRNAs and Cancer

More than 50% of miRNA genes are located in cancer-associated genomic regions or in fragile sites which suggests that miRNAs may play an important role in cancer pathogenesis [56].

The first evidence that miRNAs are involved in cancer emerged in 2002 when Calin, G.A. *et al*. found that miR-15a and miR-16–1 are downregulated or deleted in most patients with B-cell chronic lymphocytic leukemia [57]. Since then, several studies have emerged concerning the role of miRNAs in neoplastic diseases [57–60].

One of the most important features of miRNAs is that they show abnormal expression in cancerous cells in relation to normal tissues. These expression changes may occur as consequence of chromosomal abnormalities, mutations, single nucleotide polymorphisms (SNPs), epigenetic changes and defects in the miRNA biogenesis machinery (Figure 9) [57,58,60]. Furthermore, dysregulated miRNAs expression is tumor-specific and in some cases associated with diagnostic, prognostic and response to treatment [58].

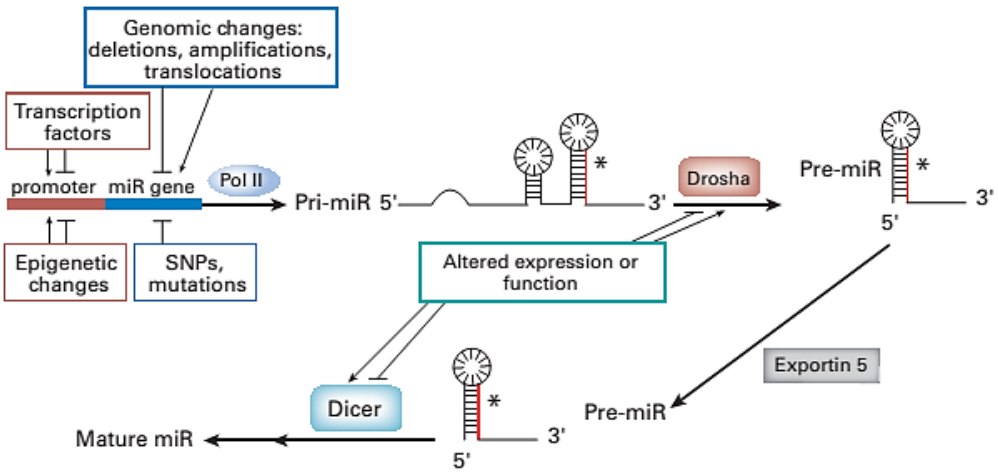

Figure 9 *- Causes for miRNAs dysregulation in cancer. Adapted from* [60].

Importantly, miRNAs affect target genes that are implicated in numerous cellular processes involved in cancer development such as proliferation, apoptosis, cell cycle, cell adhesion, angiogenesis and inflammation, becoming clear that miRNAs can function either as tumor suppressors or oncogenes (oncomiRs) depending on the functions of their target genes [59,61,62]. OncomiRs are known to downregulate tumor suppressor genes, and have been reported to be overexpressed in multiple miRNA-profiling studies [63]. On the other

hand, tumor suppressor miRNAs are responsible for downregulating oncogenes, and are mostly underexpressed in cancer [63].

Considering the importance of miRNAs in cancer biology, this is a rapidly rising field in oncology, since it holds promise for the discovery of new cancer biomarkers and new targets for the development of novel cancer therapeutics [64].

4.1.1. MicroRNAs and HPV-induced Cancers

Over the past decade, some studies have arisen indicating that miRNAs are possible biomarkers of occurrence and development of HPV-induced cancers as well as interactions between HPV oncoproteins and miRNAs have also been described [65]. It has also been confirmed that HPV is not able to express its own miRNAs, but it disturbs host miRNA expression [66].

Martinez and colleagues showed that the expression of *E6* oncogene reduced the miR-218 expression in HPV16 positive cervical carcinomas and that *LAMB3* is a possible target of miR-218 [67]. Since the LAMB3 protein increases cell migration and promotes tumorigenesis in human keratinocytes, the downregulation of miR-218 by E6 and the consequent overexpression of LAMB3 may contribute to tumorigenesis [67]. Other study from the same group demonstrated that there is a distinctly different miRNA profile in HPV-associated squamous cell carcinoma of the head and neck (SCCHN) cell lines when compared with HPV-negative SCCHN and normal oral keratinocytes cell lines [68]. They show that the expression of E6 may result in upregulation of miR-363 and downregulation of miR-181a, miR-218 and miR-29a [68].

The experiments performed by Lajer and co-workers also showed that HPV positive SCCHN have a distinct miRNA profile compared with HPV negative SCCHN and that the miRNA profile of HPV positive SCCHN and HPV positive cervical squamous cell carcinoma (CSSC) are significantly more alike compared with HPV negative SCCHN and CSSC [69]. Moreover, this group also identified a set of HPV core miRNAs such as miR-15a, miR-16, miR-143, miR-145 and the miR-106-363 cluster [69].

A study developed by Greco and colleagues showed that HPV16 E5 protein modulates the expression of miRNAs in human epithelial HaCaT cell lines and it seems to favor increased cell proliferation and tumorigenesis and, on the other hand, to repress epithelial differentiation [70].

Consequently, the study and identification of miRNAs associated with HPV-induced cancers are essential since they may be useful HPV-specific tumor biomarkers and they may prove useful in the development of future individualized treatment strategies and new targeted therapies [69].

4.2. MicroRNA-150

MiR-150 was first identified by its crucial regulatory role in normal hematopoiesis, but recent studies have shown that the dysregulation of miR-150 is frequently present not only in various types of hematological malignancies, but also in a variety of solid tumors [71,72].

Interestingly, depending on the genes that miR-150 target, it can act as an oncomiR or a tumor suppressor in both malignant hematopoiesis and solid tumors [71,72]. MiR-150 has been found to be upregulated in myelodysplastic syndrome, chronic lymphocytic leukemia, gastric cancer, breast cancer, non-small cell lung cancer and cervical cancer [71,72]. On the other hand, miR-150 is downregulated in chronic myeloid leukemia, acute myeloid leukemia, mantle cell lymphoma, conjunctival mucosa-associated lymphoid tissue lymphoma, Burkitt lymphoma, NK/T-cell lymphoma, pancreatic cancer, esophageal squamous cell carcinoma, colorectal cancer and liver cancer [71,72]. In these cancers miR-150 has multiple targets that are involved in cell differentiation, cell cycle, proliferation, apoptosis, invasion and metastasis [71,72].

Curiously, the transcriptional regulation of miR-150 in HeLa cells seems to be made by p53, which is the main target of HPV E6 oncoprotein, and Nuclear factor-kappa B (NF-kB) p65/RelA [73]. However, the expression profile of miR-150 in HPV-induced lesions remains elusive.

5. K14-HPV16 Transgenic Mice

K14-HPV16 transgenic mice *Mus musculus* are a useful experimental model to the study of epigenetic and genetic factors in HPV-induced carcinogenesis, due to the morphological and molecular similarities between these mice and humans [64].

These mice are characterized for having the early-region genes of HPV16 under control of the promoter of keratin-14 (K14) with the purpose of directing the early genes expression to basal keratinocytes, since this epidermal layer is characterized by expressing several keratins such as K14 [74,75]. There are several reports of HPV-induced malignancy in transgenic mice with *E6* and *E7* oncogenes, but these models did not targeted the oncogenes expression for keratinocytes, causing neoplastic phenotypes that are dissimilar to the multistep development of squamous epithelial neoplasia seen in clinical HPV disease [75].

There are some inbred backgrounds of K14-HPV16 mice, namely C57BL/6, BALB/c, SSIN/SENCAR and FVB/n [74]. All of these backgrounds develop hyperplastic and/or dysplastic lesions, but only in mice with the background FVB/n there is a progress to squamous cell carcinomas, which highlights the key role of host factors in the development of HPV-associated tumors [64,74]. Squamous cell carcinomas are observed on epidermis of the ear, chest and truncal skin in 21% of FVB/n mice aged 8 to 12 months [64].

Interestingly, this mouse model recapitulates multi-step carcinogenesis of the human uterine cervix [64]. In fact, a study made by Smith-McCune and co-workers have demonstrated that lesions from both K14-HPV16 and human cervix are histologically similar [76]. The hyperplastic murine lesions are histologically analogous to human cervical intraepithelial neoplasia (CIN) I lesions (mild dysplasia) and the dysplastic murine lesions are similar to human CIN III lesions (severe dysplasia to CIS) [76,77]. Murine and human invasive squamous carcinomas were both composed of clusters of malignant epithelial cells surrounded by fibrovascular stroma [76]. In the same study was also compared the patterns of angiogenesis by immunohistochemical staining [76]. The results obtained showed an upregulation of angiogenesis during the early stages of carcinogenesis in both species [76]. The high-grade premalignant lesions and neoplastic lesions in both humans and K14-HPV16 mice were characterized by an increment in the number of new capillaries and close apposition of the microvasculature to the overlying neoplastic epithelium [76]. In both species, the expression of the vascular endothelial growth factor (VEGF) was progressively upregulated during carcinogenesis [76]. The results of this study demonstrate similarities between carcinogenesis and neovascularization in the human cervix and the epidermis of K14-HPV16 mice, highlighting the significance of this model to better understand the

different players involved in the different steps of cancer progression and to determine how to target them for therapeutic applications [76].

Given the capacity of this model to reproduce the different stages of carcinogenesis, it was chosen in order to study the expression profile of miR-150 in HPV-induced lesions. In fact, other miRNAs namely miR-21 and miR-155 were already studied in lesions of these mice by Paiva and co-workers [64,78]. These studies concluded that miR-21 expression was lower in CIS (ear skin) samples compared with hyperplasia (chest skin) suggesting that tissues with lower miR-21 expression are more predisposed to cancer progression [64]. Moreover, miR-155 expression was lower in hyperplastic chest skin than in normal chest skin suggesting a possible anti-oncogenic effect of miR-155 [78].

Recently, Santos *et al.* studied the infiltration of $CD8^+$T lymphocytes in HPV-induced lesions in this model and demonstrated that celecoxib was able to promote the activation of these immune cells [79]. However, the mechanism behind this activation remained unclear [79].

6. Celecoxib

The pharmacological agent celecoxib is a specific inhibitor of the cyclooxygenase-2 (COX-2) that belongs to the class of nonsteroidal anti-inflammatory drugs [80].

COX is an enzyme which converts arachidonic acid to cyclic endoperoxidases which are then converted to prostaglandins I2, D2 and E2 and thromboxane via their respective synthases [81]. COX-2 is the inducible isoform of COX and during inflammation its expression is increased [82].

The anti-inflammatory proprieties of celecoxib are exerted by inhibiting COX-2 and consequently inhibiting the synthesis of prostaglandins which play a key role in the generation of the inflammatory response [83,84].

Celecoxib is approved by the Food and Drug Administration (FDA) for the treatment of several diseases including rheumatoid arthritis, osteoarthritis and more recently for the reduction of the number of adenomatous colorectal polyps in patients with familial adenomatous polyposis, which is a dominantly inherited syndrome of colorectal cancer predisposition [80,85,86].

Investigations have shown that celecoxib has an anti-tumor activity and a chemopreventive effect in a wide variety of tumors such as colorectal, head and neck, breast and prostate [87–94].

In fact, celecoxib interferes with tumor initiation and tumor cell growth *in vitro* and *in vivo* [95]. It has been confirmed that celecoxib induces cell cycle arrest, inhibits tumor growth, suppress tumor neo-angiogenesis and induces apoptotic cell death in tumor cells and endothelial cells [95]. Moreover, it increases the sensitivity of tumor cells to chemotherapy, radiotherapy, or chemoradiotherapy [95].

COX-2, the target of celecoxib is upregulated in a variety of human premalignant, malignant and metastatic epithelial tumors and is associated with poor prognosis [95]. In fact, COX-2 has a pro-inflammatory role which is a critical component of tumor progression since many cancers arise from sites of infection, chronic irritation and inflammation [96]. Furthermore, COX-2 expression contributes to six hallmarks of cancer (Figure 10) [81].

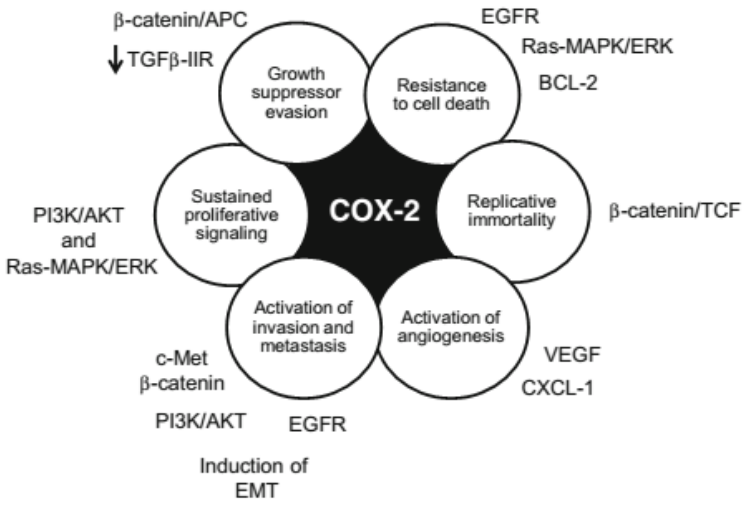

Figure 10 *- Contribution of COX-2 for the hallmarks of cancer. Adapted from:* [81].

However, it has been confirmed that celecoxib is able to suppress tumor growth without an involvement of COX-2 [95]. Actually, celecoxib has COX-2-independent molecular targets such as protein kinase B (Akt), kinase 3-phosphoinositide-dependent kinase-1 (PDK-1), CKIs and cyclins as well as the anti-apoptotic proteins surviving Bcl-2 and Mcl-1 [95].

In HPV-induced cancers such as cervical cancer, it is suggested that COX-2 induction is an early event in carcinogenesis, however it seems that celecoxib is independent of COX-2 to induce apoptosis and NF-kB appears to have a key role in celecoxib-induced apoptosis [97,98]. Moreover, it seems that celecoxib is able to restore p53, a tumor suppressor protein that is inhibited by HPV E6 oncoprotein, and consequently inhibit apoptosis [99]. Taking into account this relation between celecoxib, p53, NF-kB and HPV, it seems interesting to study if celecoxib can interfere in the miR-150 expression profile of HPV-induced lesions.

Aims of the Study

Aims of the Study

The following aims were set:

1. Main aim

- To evaluate the expression profile of miR-150 in HPV-induced lesions of K14-HPV16 transgenic mice

2. Specific aims

- To evaluate and compare the expression profile of miR-150 in chest skin samples of wild-type and K14-HPV16 transgenic mice.
- To study the expression of miR-150 along the progression of HPV-induced lesions in K14-HPV16 transgenic mice.
- To analyze the effect of celecoxib in the expression profile of miR-150 in K14-HPV16 transgenic mice.

Material and Methods

Material and Methods

1. Mice

In order to perform this study, a model of K14-HPV16 transgenic mice on a FVB/n background was used. Creation of K14-HPV16 transgenic mice has been previously reported by Arbeit *et al.* [75]. These transgenic mice were kindly donated by Dr. Jeffrey Arbeit and Dr. Douglas Hanahan, through the USA National Cancer Institute Mouse Repository. The animal experiments were approved by the University of Trás-os-Montes and Alto Douro ethics committee (10/2013) and the Portuguese Veterinary Directorate (0421/000/000/2014).

Wild-type mice and K14-HPV16 transgenic mice (Figure 11) were maintained and bred according with the Portuguese (Portaria 1005/92 dated October the 23rd) and European (EU Directive 2010/63/EU) legislation, under controlled conditions of temperature (23 ± 2°C), light–dark cycle (12h light / 12h dark) and relative humidity (50 ± 10%). Health checks were performed daily and food (4RF21 GLP, Mucedola) and water were provided *ad libitum*.

All mice were previously genotyped by our group through amplification of HPV *E6* and *E2* genes in order to assess HPV integration.

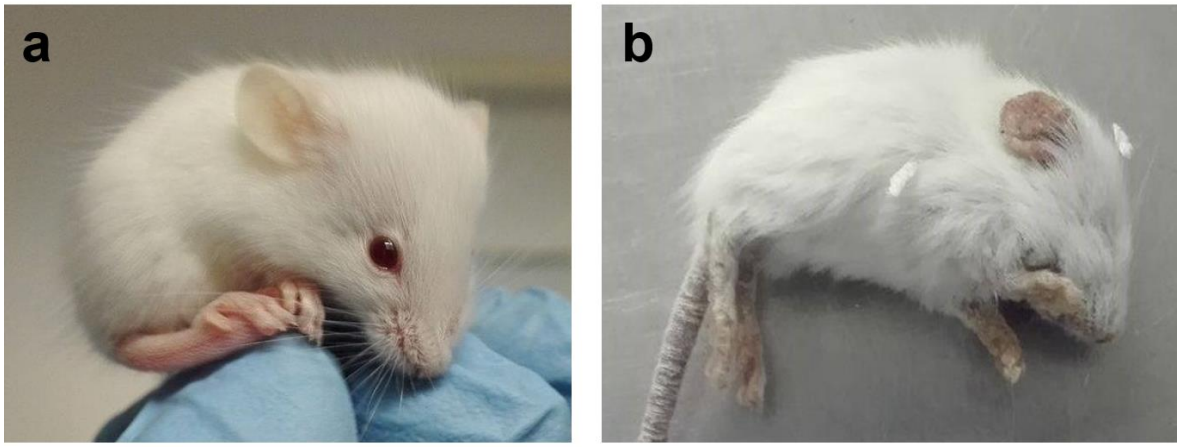

Figure 11 *- Female mice.* ***a*** *– Wild-type mouse ($HPV^{-/-}$);* ***b*** *– K14-HPV16 transgenic mouse ($HPV^{+/-}$) with hyperkeratosis and auricular erythema.*

2. Experimental Design

The present work employed archived samples from previous experimental protocols, performed for the purposes of a previous MSc thesis [79] and an ongoing PhD work. All samples were obtained from female mice ascribed to six experimental groups (Table 2). 35 K14-HPV16 transgenic ($HPV^{+/-}$) and 22 wild-type ($HPV^{-/-}$) female mice were maintained up to 24-26 weeks old and 7 $HPV^{+/-}$ and 7 $HPV^{-/-}$ females were maintained up to 28-30 weeks old in order to study different stages of carcinogenesis.

Table 2 *- Experimental groups*

Group	Genotype	Age	Treatment
1 (n = 22)	$HPV^{-/-}$	24-26 weeks	No treatment
2 (n = 21)	$HPV^{+/-}$	24-26 weeks	No treatment
3 (n = 5)	$HPV^{+/-}$	24-26 weeks	124 mg/kg/day of celecoxib
4 (n = 9)	$HPV^{+/-}$	24-26 weeks	75 mg/kg/day of celecoxib
5 (n = 7)	$HPV^{-/-}$	28-30 weeks	No treatment
6 (n= 7)	$HPV^{+/-}$	28-30 weeks	No treatment

Celecoxib (Pfizer®) was administrated orally as previously described by Santos *et al.* [79]. Matched control animals (group 2) were administrated the vehicle. Two different doses of celecoxib were given, namely 124 mg/kg/day (defined as the “high” dose) and 75 mg/kg/day (defined as the “low” dose).

3. Samples Collection

All mice were sacrificed using sodium pentobarbital followed by intracardiac puncture and exsanguination, as indicated by the Federation for Laboratory Animal Science Associations. Chest skin samples from each animal were collected into TripleXtractor reagent (Grisp®), macerated and kept at −80 °C until miRNA extraction. Matched samples were collected for histological analysis.

4. Histological Analysis

The chest skin samples collected for histological analysis were fixated in 10% neutral buffered formalin for 48h. The fixed tissues were then dehydrated through graded alcohols and xylene and embedded in paraffin using an automatic STP 120 processor

(Micro, Boise, ID). The paraffin blocks were cut into 2 µm thick sections and stained with haematoxylin and eosin (H&E) for further evaluation on a light microscope.

Samples were classified as normal skin, epidermal hyperplasia and epidermal dysplasia.

5. MicroRNAs Isolation

MiRNAs were isolated according to an optimized protocol by our group.

For miRNAs isolation it was added a chloroform solution (EMSURE®) to our samples, which after centrifugation at 12.000 g for 15 min, allows the separation of the RNA phase. Then, miRNAs were isolated using the commercial kit GRS microRNA Kit (Grisp®).

After miRNAs isolation, concentration and purity were measured for each sample using the *NanoDrop*® ND-1000 spectrophotometer. The ratio of absorbance at 260 nm and 280 nm is used to assess contamination with proteins and the ratio of absorbance at 260 nm and 230 nm is used to assess presence of contaminants as phenols.

MiRNA samples were kept at −80ºC until further use.

6. Complementary DNA Synthesis

The miRNA samples were then used as templates for complementary DNA (cDNA) synthesis using a TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems®) and sequence-specific stem-loop reverse transcription primers for miR-150 and small nucleolar RNA-202 (snoRNA-202) (Figure 12) .

The amplification conditions were as follows: 30 min at 15ºC, 52 min at 42ºC and finally 10 min at 85ºC.

cDNA was further used as template for quantitative real-time PCR (qPCR).

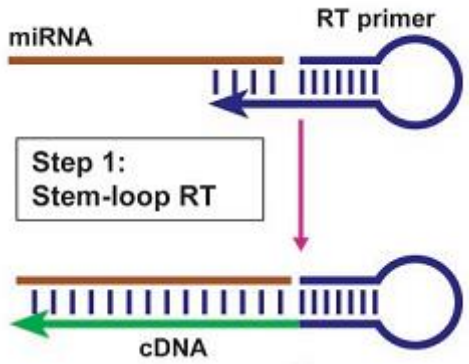

Figure 12 *- Reverse transcription of miRNA samples into cDNA by stem-loop reverse transcription primers. Adapted from:* [100].

7. Quantitative Real-Time PCR

The miRNA expression was measured by qPCR using a StepOne™ qPCR Real-Time PCR machine. The reactions were performed in a 48-well plate.

For each reaction was added 1X TaqMan® Fast Advanced Master Mix (Applied Biosystems®) with 1X probes (TaqMan® microRNA Expression Assays, miR-150: 000473, Applied Biosystems® or TaqMan® microRNA Expression Assays, snoRNA-202: 001232, Applied Biosystems®) and cDNA sample making a total volume of 10µl (Figure 13).

To normalize the results, snoRNA-202 was used as endogenous control. SnoRNA-202 is one of the recommended endogenous controls for miRNA expression data analysis, because it demonstrates the highest abundance and least variability in a wide variety of tissues and cell lines in mouse [101]. This endogenous control was also previously tested by our group.

The miRNAs quantification was performed in duplicate and negative controls lacking cDNA were included in all reactions.

The thermal cycling conditions were as follows: 10 min at 95ºC followed by 45 cycles of 15 seconds at 95ºC and 1 min at 60ºC.

Data analysis was made using StepOne™ Sofware v2.2 (Applied Biosystems®) with the same baseline and threshold set for each plate, in order to generate threshold cycle (Ct) values for miR-150 or snoRNA-202 in each sample. The results were confirmed by two independent investigators.

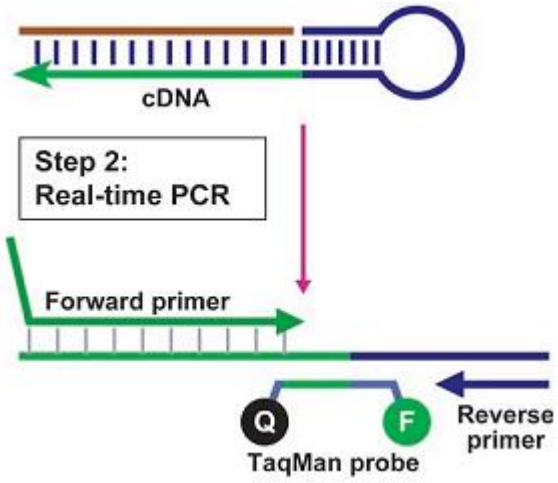

Figure 13 *- qPCR using a cDNA sample, forward primer, reverse primer and a dye-labeled TaqMan probe. Adapted from:* [100].

8. Statistical Analysis

Statistical analysis was performed using the statistical software SPSS for Windows (Version 18.0) (IBM® SPSS®).

T-Student's test was used in order to evaluate any statistical differences in normalized relative expression (−ΔCt) of miR-150 between the different groups.

Results were considered statistically significant when *p* values were less than 0.05.

Results

Results

1. Histological Analysis

Evaluation of the skin samples of $HPV^{-/-}$ mice (negative control) on a light microscope, showed that these animals presented normal skin histology at both 24-26 weeks old and 28-30 weeks old (Figure 14a and Table 3).

Analyzing skin samples of $HPV^{+/-}$ mice at 24-26 weeks of age it was possible to observe epidermal hyperplasia characterized by an increase of epidermal thickness, increase of the number of cell layers, presence of papillomatosis, hyperkeratosis and inflammation in 100.0% of the animals. Dysplastic lesions were observed in 31.8% of the animals (Figure 14b and Table 3).

In $HPV^{+/-}$ mice at 28-30 weeks of age it was observed hyperplastic lesions in 50% of the animals, and dysplastic lesions characterized by the disorganization of the epidermis, presence of papillomatosis, hyperkeratosis, mitotic cells, inflammatory cell infiltrates and an increase of neo-vascularization in 100% of the animals (Figure 14c and Table 3).

In $HPV^{+/-}$ mice treated with 124 mg/kg/day of celecoxib the incidence of epidermal dysplasia was not observed, however in $HPV^{+/-}$ mice treated with 75 mg/kg/day of celecoxib, it was observed dysplastic lesions in only one mice, which corresponds to 12.5%. In all mice treated with the two doses of celecoxib, it was observed epidermal hyperplasia (Table 3).

CIS and invasive carcinoma were not observed in our samples.

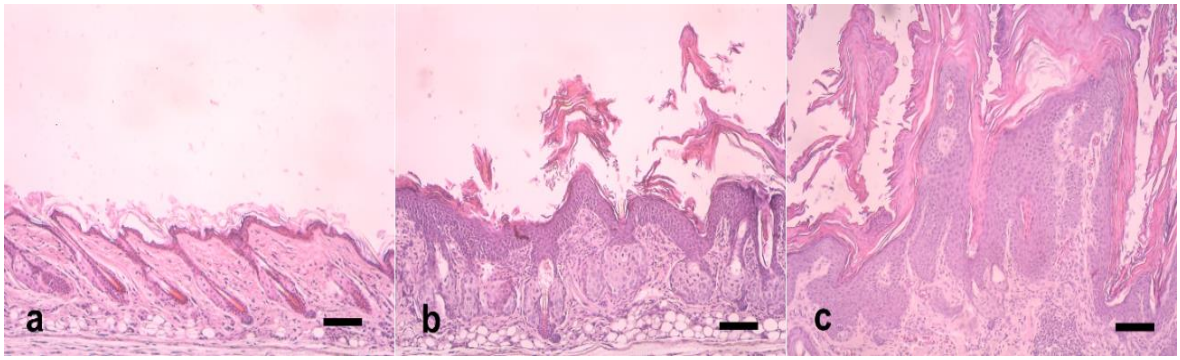

Figure 14 *- Histological Analysis of mice skin samples, H&E, 100x.* ***a*** *– Wild-type mice ($HPV^{-/-}$) showing normal skin;* ***b*** *– K14-HPV16 transgenic mice ($HPV^{+/-}$) with epidermal hyperplasia;* ***c*** *– $HPV^{+/-}$ mice with epidermal dysplasia*

Table 3 *- Histological classification of mice skin samples*

Group	Age	Treatment	Cutaneous lesions incidence (%)		
			Normal Skin	Epidermal hyperplasia	Epidermal dysplasia
1 ($HPV^{-/-}$, n = 22)	24-26 weeks	No treatment	22/22 (100%)	0/0 (0%)	0/0 (0%)
2 ($HPV^{+/-}$, n = 21)	24-26 weeks	No treatment	0/0 (0%)	22/22 (100%)	7/22 (31.8%)
3 ($HPV^{+/-}$, n = 5)	24-26 weeks	124 mg/kg/day of celecoxib	0/0 (0%)	5/5 (100%)	0/0 (0%)
4 ($HPV^{+/-}$, n = 9 *)	24-26 weeks	75 mg/kg/day of celecoxib	0/0 (0%)	8/8 (100%)	1/8 (12.5 %)
5 ($HPV^{-/-}$,n = 7)	28-30 weeks	No treatment	7/7 (100%)	0/0 (0%)	0/0 (0%)
6 ($HPV^{+/-}$, n= 7 *)	28-30 weeks	No treatment	0/0 (0%)	3/6 (50%)	6/6 (100%)

***** In one of the cases no tissue was available to histological analysis

2. MicroRNA-150 Expression

2.1. MiR-150 is overexpressed in HPV-induced lesions of HPV$^{+/-}$ mice

To evaluate the miR-150 expression profile in HPV-induced lesions, miR-150 levels were measured by qPCR in HPV-induced chest skin lesions of HPV$^{+/-}$ mice and in chest skin of HPV$^{-/-}$ mice. The miR-150 expression levels were significantly increased in lesions from HPV$^{+/-}$ mice when compared with normal skin from to HPV$^{-/-}$ mice ($p < 0.001$) (Figure 15).

MiR-150 levels were also compared between chest skin tissue of HPV$^{-/-}$ mice and HPV$^{+/-}$ mice at 24-26 weeks of age and HPV$^{-/-}$ and HPV$^{+/-}$ mice at 28-30 weeks of age. The results obtained were consistent with the previous ones, showing that miR-150 is significantly overexpressed in HPV-induced lesions of HPV$^{+/-}$ mice when compared with HPV$^{-/-}$ mice in both 24-26 weeks old and 28-30 weeks old mice ($p = 0.024$ and $p = 0.001$, respectively) (Figure 16).

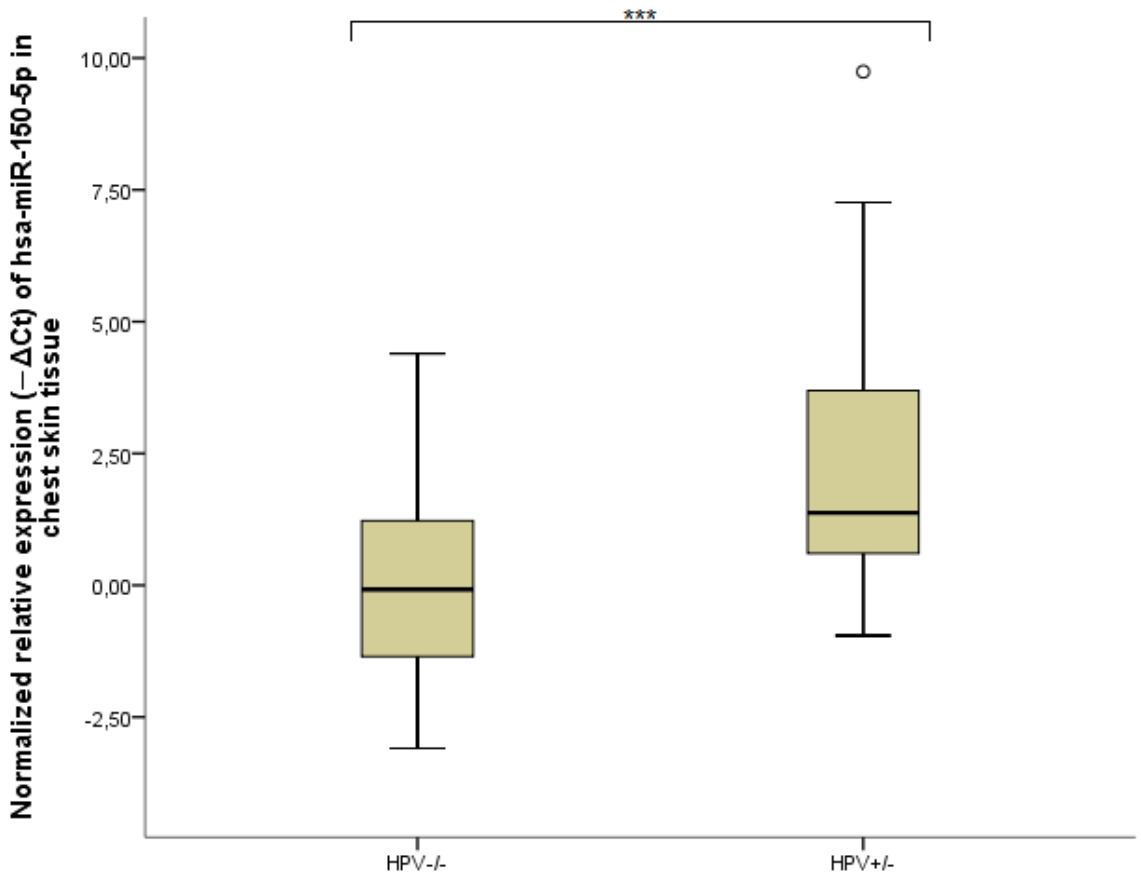

Figure 15 *- Normalized relative expression of miR-150 in chest skin tissue of wild-type mice (HPV$^{-/-}$) and K14-HPV16 transgenic mice (HPV$^{+/-}$). *** p < 0.001.*

2.2. MiR-150 expression increases along with the severity of HPV-induced lesions

With the purpose of comprehending if the expression level of miR-150 correlates with the progression of the lesions and severity, miR-150 expression in $HPV^{+/-}$ 24-26 weeks old and 28-30 weeks old mice was compared. Our data suggest a trend for miR-150 expression level to be significantly increased in the more advanced lesions of older $HPV^{+/-}$ mice compared with earlier lesions from younger mice (p = 0.001) (Figure 16).

In order to study whether the difference in miRNA expression levels between these two groups was in fact due to lesions progression, we studied the expression level of miR-150 in 24-26 weeks old and 28-30 weeks old $HPV^{-/-}$ mice. There were no statistical differences between groups (p = 0.104) (Figure 16).

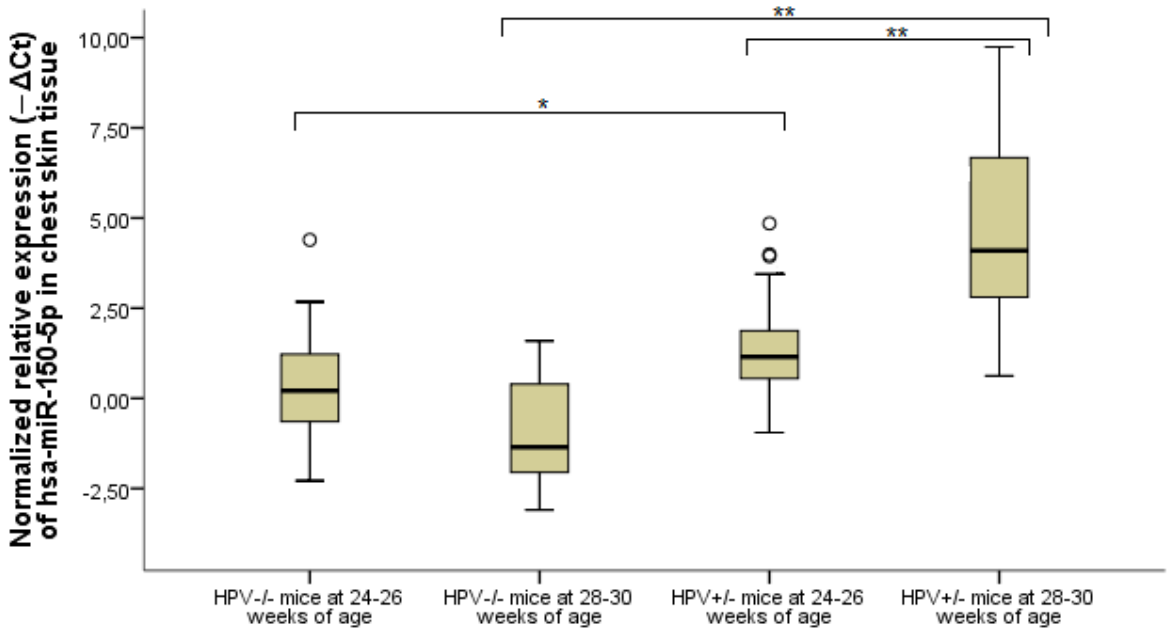

Figure 16 *- Normalized relative expression of miR-150 in chest skin tissue of wild-type mice ($HPV^{-/-}$) at 24-26 and 28-30 weeks old and K14-HPV16 mice ($HPV^{+/-}$) at 24-26 and 28-30 weeks old. * $p < 0.05$; ** $p < 0.01$.*

2.3. Celecoxib influences the expression profile of miR-150 in HPV-induced lesions

The miR-150 expression profile in untreated HPV$^{+/-}$ mice at 24-26 weeks of age and age-matched mice treated with a “high” and “low” dose (124 mg/kg/day and 75 mg/kg/day respectively) of celecoxib was studied. The results showed that the high-dose of celecoxib significantly decreased the expression of miR-150 in HPV-induced lesions when compared with HPV-induced lesions from matched control mice ($p < 0.001$). No statistical differences were observed between HPV$^{+/-}$ mice treated with low-dose of celecoxib and HPV$^{+/-}$ mice with no treatment ($p = 0.114$) (Figure 17).

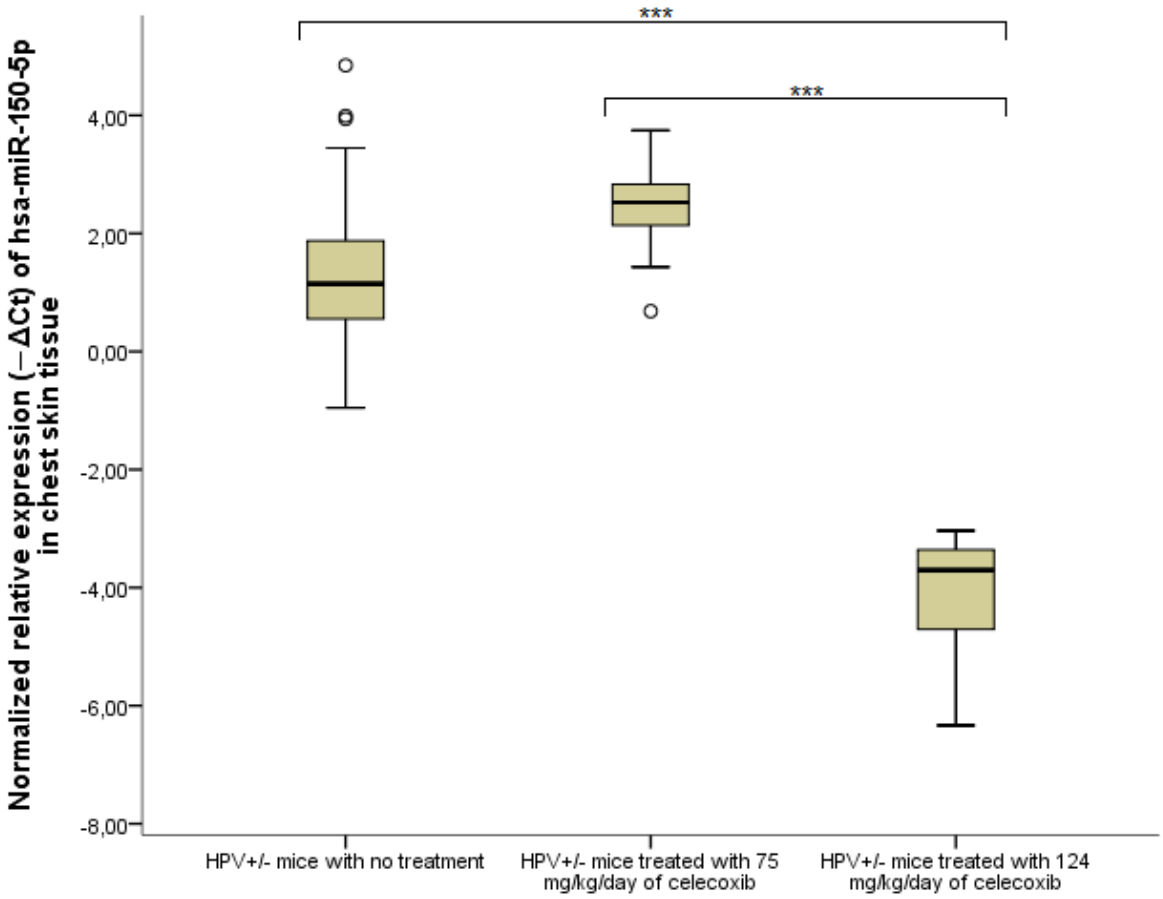

Figure 17 *- Normalized relative expression of miR-150 in chest skin lesions of K14-HPV16 mice (HPV$^{+/-}$) with no treatment, treated with a low-dose of celecoxib (75 mg/kg/day) and treated with a high-dose of celecoxib (124 mg/kg/day). *** p < 0.001.*

Discussion

Discussion

Nowadays, high-risk HPV is a firmly established etiological agent of several cancers including cervical cancer, anogenital cancer and some head and neck squamous cell carcinomas, and has become a major health problem worldwide, particularly in developing countries [102]. Although the persistent HPV infection has long been established as necessary, the infection by itself may not be sufficient to induce malignant transformation [103]. Therefore, in view of the growing need to understand the various factors involved in HPV-induced carcinogenesis, the development of experimental models becomes an essential tool. The development of K14-HPV16 transgenic mice allows to identify and study genetic and epigenetic factors involved in all the stages of the multistep carcinogenesis induced by HPV, with high similarities to the HPV-induced carcinogenesis of human cervix [74,76].

In recent years, there has been a growing interest and investment in oncology, notably in identifying and defining molecular biomarkers of risk for cancer development. Particularly, the study of miRNAs in tumor biology is an area of rapid growth in cancer research since they have been identified as potential prognostic biomarkers and possible targets for the development of new therapies [104]. Recent studies have significantly enhanced the complexity of HPV-induced carcinogenesis with the discovery of miRNAs as important players at regulating a variety of pathways [68,69,105,106]. Since miRNAs dysregulation is seen in precancerous lesions, they hold promise as early detection biomarkers or even therapeutic targets [107]; however, the number of studies that have explored these issues are still scarce and sometimes with conflicting results.

MiR-150 expression has been found to be dysregulated in a variety of solid cancers such as cervical, breast, lung, colorectal, gastric, liver, pancreatic and esophageal cancer, however its expression profile in HPV-induced lesions remains elusive [108–116]. In the present study, it was observed that miR-150 is significantly overexpressed in HPV-induced chest skin hyperplastic and dysplastic lesions of K14-HPV16 transgenic mice ($HPV^{+/-}$) when compared with chest skin tissue of wild-type mice ($HPV^{-/-}$). Since $HPV^{+/-}$ mice at 28-30 weeks of age present more aggressive HPV-induced lesions and higher levels of miR-150 when compared with $HPV^{+/-}$ mice at 24-26 weeks of age, it seems that mir-150 expression is associated with the severity of HPV-induced lesions. In our samples, CIS and invasive carcinoma were not observed, because these stages are only present in older animals [74]. Recent studies have documented the aberrant expression of miR-150 associated with cancer development and progression through the regulation of oncogenes or tumor suppressor genes [71,72]. In cervical cancer, miR-150 has been shown to be upregulated when compared with normal cervical tissues and para-carcinoma tissues [108]. A previous

study by Li *et al.* demonstrates that high levels of miR-150 promote cell growth and survival by targeting FOXO4, resulting in a decrease of p27, FASL and BIM mRNA and protein levels and in an increase of mRNA and protein levels of cyclin D1 (Figure 18) [108]. This study also shows that the stage progression is correlated with an increase of miR-150 expression [108]. This is in agreement with our data since we verified that miR-150 is overexpressed in HPV$^{+/-}$ mice and that the expression level of miR-150 is increased along with HPV-induced lesions progression.

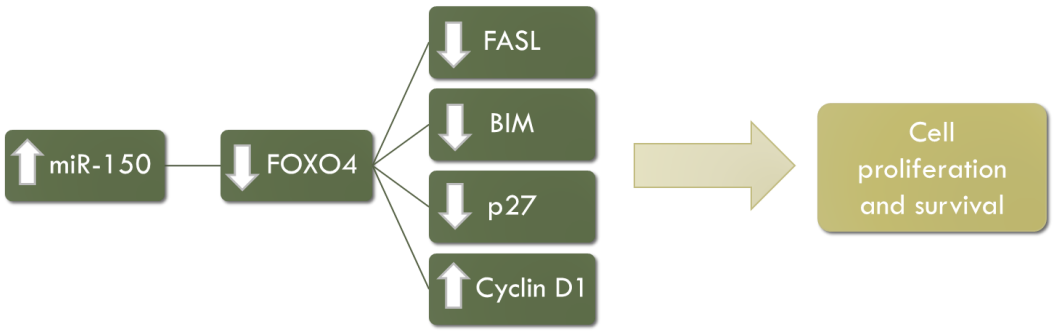

Figure 18 *– Schematic illustration of how miR-150 promotes cervical cancer cell growth and survival.*

The oncogenic proprieties of high-risk HPV are mainly due to the expression of E6 and E7 oncoproteins which lead to the inactivation of p53 and pRB respectively (Figure 19) [37]. A previous study by Ghose *et al.* in human cervical carcinoma HeLa cells, identified p53 as a transcription factor that binds to the upstream sequence of miR-150 regulating its expression. In the same study, it is reported that when p53 is knocked down in HeLa cells, the expression of miR-150 is significantly increased (Figure 19) [73]. These results also support our findings since our mouse model expresses the early-region genes of HPV16, including *E6* and *E7* oncogenes in the basal keratinocytes [74].

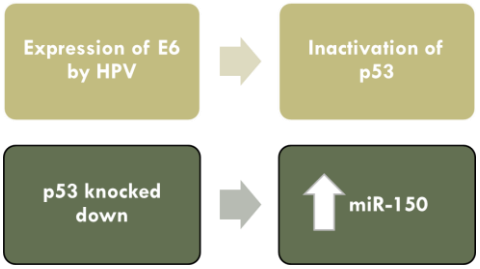

Figure 19 *– Hypothetical explanation for miR-150 overexpression in HPV$^{+/-}$ untreated mice.*

Recently, some studies have reported that miR-150 is highly expressed in other carcinomas. In breast cancer, upregulation of miR-150 is correlated with growth and proliferation and may play a role in the progression of breast cancers [109]. This study by Huang *et al.* showed that miR-150 inhibitors induce apoptosis in breast cancer cell lines while ectopic expression of the miR-150 results in increased cell proliferation [109]. $P2X_7$, a receptor that regulates cell growth through mediation of apoptosis, it is also identified as a new miR-150 target, since miR-150 levels are inversely correlate with $P2X_7$ in breast cancer cell lines and carcinomas, and that the 3′-UTR of $P2X_7$ contains a highly conserved miR-150-binding site [109]. In gastric cancer cell lines and tissues, miR-150 overexpression seems to promote cell proliferation and growth by targeting the pro-apoptotic gene early growth response factor 2 (EGR2) [113]. Furthermore, in lung cancer it is also reported that miR-150 is upregulated and represses the SRC kinase signaling inhibitor 1 (SRCIN1) by directly recognize its 3'-UTR [111]. Repression of SRCIN1 eventually promoted the proliferation and migration of lung adenocarcinoma A549 cells [111].

Celecoxib is an anti-inflammatory drug and exerts its function by inhibiting COX-2 and consequently inhibiting the synthesis of prostaglandins which play a key role in the generation of the inflammatory response [80,83,84]. It has been proven that celecoxib has an anti-tumor and chemopreventive effect through COX-2 dependent or independent mechanisms in a wide variety of tumors such as colorectal, head and neck, breast and prostate [87–95]. Regarding our study, it was observed that $HPV^{+/-}$ mice treated with 124 mg/kg/day of celecoxib, have lower miR-150 expression when compared with not treated $HPV^{+/-}$ mice. To the best of our knowledge, this is the first study reporting the influence of celecoxib in the downregulation of miR-150. To explain our results, we may consider several research lines.

Firstly, a reported study by Saha *et al.* indicates that celecoxib induces apoptosis by restoring p53 in cervical cancer cells infected with HPV18 [99]. The restoration of p53 by celecoxib can be achieved by multiple molecular mechanisms, namely: inhibition of HPV E6 oncoprotein which is essential for the stabilization and accumulation of p53; inhibition of COX-2 increases the transcription of p53 and prevents the association between COX-2 and p53 protein, which allows p53 translocation to the nucleus; and activation of the protein kinase ATM and p38MAPK which mediate p53 phosphorylation resulting in a transcriptionally active p53 (Figure 20) [99]. The study by Ghose *et al.* reported that in the presence of exogenous p53, miR-150 is significantly downregulated (Figure 20) [73].

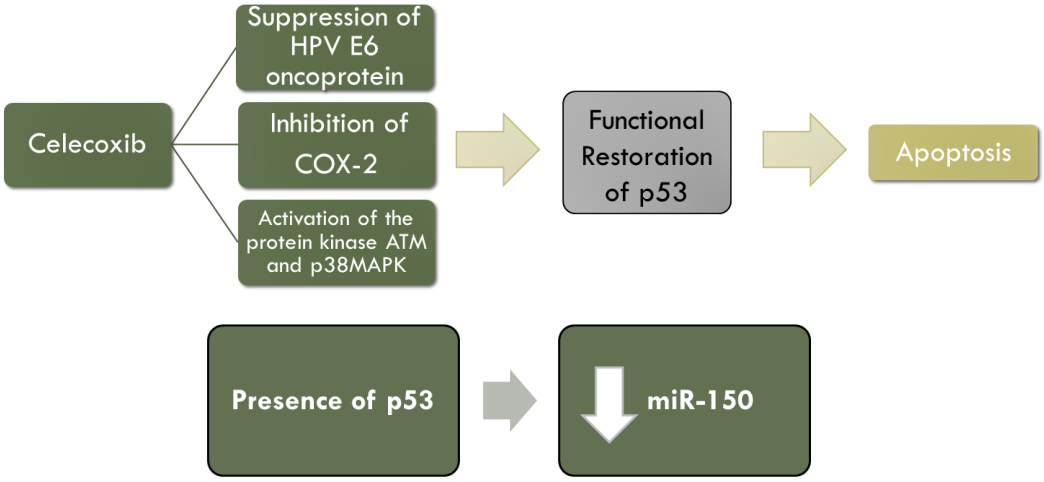

Figure 20 *- Schematic illustration of the first hypothetical explanation for miR-150 downregulation in HPV$^{+/-}$ mice treated with celecoxib.*

Secondly, the study by Ghose *et al.* also identified NF-kB p65/RelA as a transcription factor of miR-150 in human cervical carcinoma HeLa cells [73]. This study also showed that the expression levels of miR-150 decreased significantly in HeLa cells treated with acetylsalicylic acid (Aspirin®), due to the capacity of aspirin to inhibit NF-kB p65/RelA activity (Figure 21) [73]. Furthermore, it is reported that celecoxib also has the capacity of inhibiting tumor necrosis factor alpha (TNFα)-induced NF-kB activation in a dose-dependent manner (Figure 21) [117]. In fact, a study by Wang *et al.* shows that the TNFα-induced NF-kB activation upregulates miR-150 expression (Figure 21) [118].

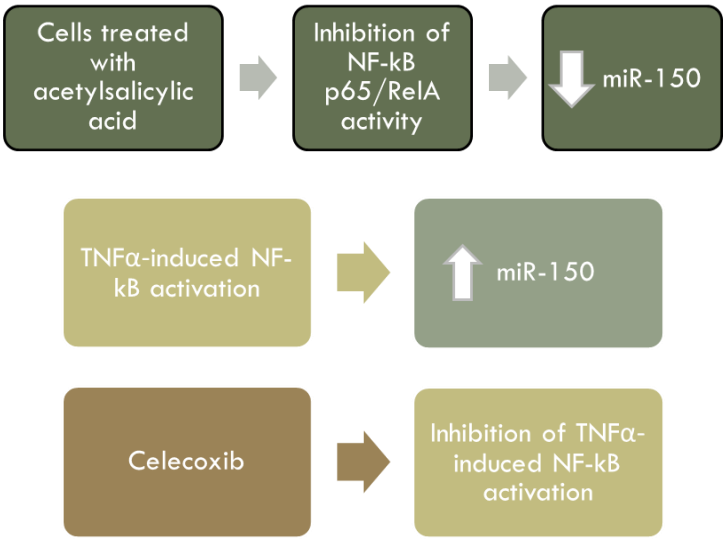

Figure 21 *- Schematic illustration of the second hypothetical explanation for miR-150 downregulation in HPV$^{+/-}$ mice treated with celecoxib.*

All these findings together may explain why we verified that miR-150 is overexpressed in HPV-induced lesions, and why miR-150 is significantly downregulated in $HPV^{+/-}$ mice treated with 124 mg/kg/day of celecoxib. Moreover our results also may give some light on the activation of $CD8^{+}$T lymphocytes by celecoxib in HPV16-induced lesions in this model, recently reported by our group, since miR-150 downregulation is essential for $CD8^{+}$T cells activation [79,119,120].

Conclusions and Future Perspectives

Conclusions and Future Perspectives

With the present findings, it is possible to conclude that the overexpression of miR-150 occurs in HPV-induced lesions in this model. Its expression seems to increase along with the severity of the lesions, reinforcing the hypothesis that miR-150 may act as an oncomiR in HPV-induced lesions. We also show that the expression profile of miR-150 is altered in the presence of high-dose (124 mg/kg/day) of celecoxib, indicating that this miRNA may be a potential biomarker for celecoxib therapy. The effect of celecoxib seems to be dose-dependent.

However, more studies are needed in order to better comprehend the mechanisms involved in miR-150 expression and its role in HPV-induced carcinogenesis. In the future, it will be interesting to study the expression profile of miR-150 in HPV-induced malignant lesions. It will be also important to study the targets of miR-150 in our $HPV^{+/-}$ samples and understand if miR-150 levels are associated with apoptosis or inflammation in HPV-induced lesions.

Moreover, it will be interesting to better comprehend the mechanisms underlying the modulation of miR-150 by celecoxib. For that purpose, it would be useful to compare the p53 and NF-kB status between non-treated and treated samples.

The study of the different molecular factors involved in HPV-induced carcinogenesis will allow the identification of new targets and consequently the putative development of new and more efficient therapies.

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